Glutamine-330 is not essential for activity in isopenicillin N synthase from *Aspergillus nidulans*

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Abstract The non-heme ferrous dependent oxidase isopenicillin N synthase (IPNS) catalyses the biosynthesis of isopenicillin N from a tripeptide substrate. The crystal structure of *Aspergillus nidulans* IPNS complexed to manganese reveals a six co-ordinate metal ligated by two water molecules and four protein ligands: His-214, His-270, Asp-216 and Gln-330 (the penultimate C-terminal residue). Modification of Gln-330 to Ala or Leu, or deletion of 2 or 6 residues from the C-terminus resulted in lowering of specific activity; no activity was observed after deletion of 8 residues. The results demonstrate that metal ligation by Gln-330 is not required for catalytic activity.

Key words: Isopenicillin N synthase; L-δ-(α-Aminoadipoyl)-L-cysteinyl-D-valine; Oxidase; Dioxygenase; Penicillin

1. Introduction

Isopenicillin N synthase (IPNS) catalyses the biosynthesis of isopenicillin N, the precursor of all other penicillins and cephalosporins, by the oxidative bicyclisation of the tripeptide, L-δ-(α-aminoadipoyl)-L-cysteinyl-D-valine (ACV) (see scheme below). IPNS is a member of an extended family of non-heme oxidases and oxygenases which all have a requirement for ferrous iron [1,2].

Spectroscopic studies have demonstrated the presence of an aspartyl and two or three histidinyl residues ligating iron at the active site of IPNS [3]. It has also been suggested that one of the histidinyl ligands might be displaced upon binding of ACV [3,4]. The spectroscopic assignments of an aspartyl and two histidinyl ligands were supported by mutagenesis studies in the light of comparisons of IPNS sequences with those of related oxygenases [5,6]. The crystal structure of IPNS (*Aspergillus nidulans*[1]) complexed to manganese substituting for iron revealed a 6 co-ordinate metal ligated by two water molecules and four protein ligands: His-270, His-214, Asp-216 and the unexpected fourth ligand, Gln-330, the penultimate residue on the C-terminus of IPNS [7].

Comparison of the last eight C-terminus residues of IPNSs from different organisms (with the sole exception of IPNS from *Streptomyces cattleya*) reveals a region of close similarity, in which the penultimate glutaminyl residue is conserved (Table 1). The DNA sequence of IPNS from *S. cattleya* [Wang, Y. and Li., R., unpublished data (Swissprot database, accession number D78166)] encodes IPNS with an apparently truncated C-terminus. If the *S. cattleya* sequence does indeed encode a catalytically active IPNS, it would seem likely that ligation of the active site iron by Gln-330 is not essential for catalytic activity of IPNS. Herein, we report the results of a study which explores the importance of Gln-330 for catalytic activity of IPNS (*A. nidulans*). The results clearly demonstrate that neither Gln-330 nor the C-terminal carboxylate of Thr-331 are essential for catalytic activity.

2. Materials and methods

2.1. Materials

DNA manipulations were carried out essentially according to Sambrook et al. [8]. Enzymes for molecular biology were purchased from Pharmacia, Promega or New England Biolabs. Double-stranded DNA sequencing was carried out using Sequenase 2.0 according to the manufacturer's instructions (US Biochemical Corp.).

The original wild-type IPNS (*A. nidulans*) expression plasmid, pJB703, used throughout this study was constructed by J.M. Blackburn, and the expression and growth of clones containing wild-type and mutant genes were carried out in *Escherichia coli* strain NM554 as previously described [9].

Site-directed mutagenesis was carried out using the Pharmacia USE kit according to the manufacturer's instructions. Stop codons were introduced at residues 323, 325 and 329 to obtain truncations in the C-terminus or modifications of glutamine were made by substituting the appropriate codon to attain the desired mutations (Table 1).

The Mcat mutant, in which the C-terminus of IPNS (*A. nidulans*) was modified to that of IPNS (*S. cattleya*), was constructed by insertion of a synthetic cassette corresponding to the truncated C-terminus of *S. cattleya* (forward strand: 5′-GT GAC TAT CTG CAG GAA GGT TTC ACT CGT TAG-3′; reverse strand: 5′-CG CGC TAA CGC GTG AAA CCT TCC TGC AGA TA-3′).

The pJB703 plasmid contains a single *Hin*dIII restriction site which was exchanged with an *Mlu*I site using the following selection primers: *Hin*dIII to *Mlu*I: 5′-GGC TGC AGC CAC GCG TGG CTG TTT TGG-3′; *Mlu*I to *Hin*dIII: 5′-GCC AAA ACA GCC AAG CTT GGC TGC AGC C-3′. The appropriate mutagenised region was se-

Table 1
Synthetic oligonucleotides used for mutagenesis

Mutant	Target primer
Q330A	5′-AAC AAG AAC GGC GCT ACC TAG AAG CGA GGG-3′
Q330L	5′-AAC AAG AAC GGC CTG ACC TAG AAG CGA GGG-3′
Q330D	5′-AAC AAG AAC GGC CTG ACC TAG AAG CGA GGG-3′
G329	5′-ATC AAC AAG AAC GGC TAG ACC TAG AAG CGA-3′
I325	5′-CTG GTG AGT TTG ATC TAG AAG AAC GGC CAG-3′

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Abbreviations: DTT, dithiothreitol; ACV, L-δ-(α-aminoadipoyl)-L-cysteinyl-D-valine; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; IPNS, isopenicillin N synthase.

[1]Also known as *Emericella nidulans*.

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quenced in both directions by Sanger's dideoxy chain termination method to verify the specific mutations at the site desired.

Sequence alignments were performed using software from the Genetics Computer Group [10].

2.2. Expression and enzyme purification

Recombinant cells carrying the wild-type or mutant IPNS (*A. nidulans*) plasmids were used to inoculate flasks of 2×TY medium containing 35 μg/ml chloramphenicol. After overnight growth at 37°C cells were harvested by centrifugation and stored at −80°C. Two different purification procedures were applied depending upon whether the enzyme was to be used for hole plate (1) or kinetic assays (2):

(1) Cells were sonicated in lysis buffer (50 mM Tris pH 8.0, glycerol 10% w/v, 5 mM EDTA, 0.1% Triton X-100, 0.1% β-mercaptoethanol) and the crude cell lysate was applied to a 1 ml Resource Q column (Pharmacia). Fractions were collected from the column using a salt gradient (20 ml, 0–0.5 M NaCl in 50 mM Tris pH 8.0) and IPNS was eluted at approx. 150 mM NaCl. All protein-containing fractions were analysed by SDS-PAGE and the two purest fractions (by SDS-PAGE analysis) were pooled and desalted using a PD10 column (Pharmacia) into low salt buffer (10 mM Tris pH 8).

(2) IPNS was purified from crude cell lysate using a three column purification protocol as described elsewhere [11].

All protein purification procedures were performed at 4°C and all the purified protein was stored at −80°C with little loss of activity for up to 6 months.

2.3. Assays for IPNS activity

The specific activities of purified IPNSs were determined by the hole plate assay using *Staphylococcus aureus* N.C.T.C. 6571 as the indicator organism [9]. Apparent K_m and k_{cat} values were determined by monitoring isopenicillin N production by HPLC analysis. The standard assay mixture contained in a final volume of 100 μl, 2 mM ACV, 4 mM DTT, 1mM ascorbate, 0.10 mM $FeSO_4$, 50 mM Tris pH 8.0 and up to 100 μM (3.6 mg/ml) apo-enzyme. The appearance of isopenicillin N was linear with respect to time for up to 20 min under the conditions employed. For kinetic assays by HPLC, incubations were terminated after 5 min at 28°C by addition of an equal volume of methanol. The samples were centrifuged for 5 min at 11 000×*g* to remove any denatured protein and analysed immediately by HPLC or stored at −20°C. HPLC analysis was carried out using an ODS C18 column (5 μm, 250 mm×4.6 mm) and the mobile phase for optimal separation was a gradient of 2.5–45% acetonitrile in 50 mM potassium phosphate pH 6.8. UV detection was at 220 nm at a sensitivity of 0.04 AUFS. Protein concentrations were determined by the method of Bradford [12] using BSA as standard. The amount of isopenicillin N produced in the HPLC assays was quantified by comparison with a standard of synthetic isopenicillin N, calibrated by ^{1}H-NMR (500 MHz) analysis.

2.4. Spectroscopy

CD measurements in 50 mM sodium phosphate pH 8.0 at a protein concentration of 5.3 μM were carried out in a 1 mm path length cell using a Jasco 720 spectropolarimeter operated at a scan rate of 50 nm/min in the range 195–250 nm. Mass spectrometric analyses were performed using the electrospray ionisation technique on a BIO-Q Triple Quad mass spectrometer (VG instruments). Typically, 75–150 pmol of protein were dissolved in 15 μl of 50% acetonitrile in water and injected into the electrospray ionisation source. Scans of 3 s were accumulated covering a 800–1500 Da range and spectra were analysed employing the Mass Lynx 2.0 software (VG organic). Calibrations were performed separately by the injection of horse heart myoglobin.

3. Results

3.1. Expression and purification of wild-type IPNS and its mutants

Overnight growth at 37°C of wild-type or mutant IPNS cultures resulted in the overexpression of a soluble 36 kDa protein (approx. 40% of total soluble protein as determined by SDS-PAGE analysis) as previously reported [9]. Purification protocol 1 gave 5–10 mg of protein from 100 ml overnight culture which was >90% pure by SDS-PAGE analysis and was suitable for molecular weight determinations by ESIMS or for activity determination by hole plate assay. All protein preparations from three column purification (protocol 2) resulted in IPNS of >95% purity as determined by SDS-PAGE analysis (Fig. 2).

Fig. 1. Sequence comparison of the C-terminal regions of IPNSs from sequence databases (with accession numbers in parentheses) together with IPNS mutants. The last row shows identity/similarity of the C-terminus amino acids: totally conserved (bold capitals); significantly conserved (capitals); non-conserved residues (lower case). The Gln-330 is also shown in bold. The bar indicates residues which form part of the C-terminal α-helix (α-10, shaded) and β-sheet (β-16, black) based on the Mn(II) IPNS structure [7].

Table 2
Hole plate assays and mass spectrometric analyses of IPNS (*A. nidulans*) and mutants

Enzyme type	Calculated[a] molecular mass (Da)	Observed molecular mass (Da)	Relative specific activities (wild type = 100%)
Wild type	37 391	37 392 (±1)	100
Q330A	37 334	37 335 (±1)	63
Q330L	37 376	37 381 (±2)	100
Q330D	37 378	37 382 (±2)	64
G329	37 161	37 161 (±3)	40
I325	36 748	36 746 (±2)	10
Mcat	36 652	36 653 (±4)	<0.5

[a]Values calculated from translated amino acid sequences based on reported DNA sequences.

3.2. Spectroscopy

The molecular masses from ESIMS of the mutant IPNSs (*A. nidulans*) correlated well with the values from the predicted amino acid sequences (Table 2). Analysis by CD spectroscopy showed no significant differences between wild-type IPNS (*A. nidulans*) and either active or inactive mutant enzymes (data not shown).

3.3. Hole plate and kinetic assay

Enzyme assays were carried out (at least) in duplicate using (at least) two different protein concentrations of each mutant enzyme with essentially the same results. The Mcat mutant showed no activity by hole plate assay method (Table 2); hole plate assays repeated with higher protein concentrations (up to 100 μM) also failed to show activity with this protein.

The trends in the observed specific activities for the other IPNS (*A. nidulans*) mutants from the hole plate assay qualitatively correlated well with the HPLC assays (Table 3). The HPLC assays were carried out (at least) in duplicate on each mutant IPNS with essentially the same results and the measurements varied no more than 20% between two independently purified batches of the same protein. The specific activity for the recombinant wild-type IPNS (*A. nidulans*) using the HPLC assay was somewhat lower than that reported for IPNS (*C. acremonium*) enzyme using an oxygen electrode [13] assay or an ACV consumption assay [5].

4. Discussion

The crystal structure of IPNS complexed to manganese shows the C-terminal α-helix projecting between two of the eight β-strands forming the 'jelly roll' β-barrel of IPNS. The eight residues subsequent to the C-terminal helix form a loop terminating in the conserved (except apparently for the *S. cattelaya* IPNS) Gln-330–Thr-331 dipeptide. Ligation of the cysteinyl thiol of ACV to the active site iron has been demonstrated by spectroscopic studies [14,15]. Analysis of the IPNS-manganese structure suggests that this cannot occur without prior displacement of at least one of the four metal ligands (His-214, Asp-216, His-270, Gln-330). Since the two histidinyl and aspartyl residues form part of the jelly roll platform of IPNS and are conserved throughout other sequence related members of the family, we have proposed that the amide side chain of Gln-330, which is only conserved through IPNS isozymes, is displaced from the iron during catalysis [7].

The modified IPNSs in which Gln-330 was substituted by alaninyl (Q330A) or leucinyl (Q330L) residues display reduced but substantial catalytic activities of greater than 50% of that of the wild-type enzyme. Deletion of two C-terminal residues (G329) also led to an approx. 50% reduction in specific activity. Hence, ligation of the amide side chain of the glutaminyl residue to the active site metal is clearly not essential for catalysis (Tables 2 and 3). Since Gln-330 is the penultimate residue on the C-terminus of IPNS it is possible that in the Q330A, Q330L and G329 mutants the role of the amide side chain of Gln-330 might be fulfilled by the C-terminal carboxylate. However, a further C-terminally truncated version of IPNS with six residues deleted (I325) was still active, albeit with a very much reduced specific activity of only approx. 10% of that of the wild-type enzyme.

In order for the C-terminus of the I325 mutant to reach to the active site metal (minimally) some unravelling of the C-terminal α-helix must occur. Since this is unlikely to occur without a distortion of the structure, the possibility of the C-terminal carboxylate substituting for the amide side chain of Gln-330 in the modified IPNS is ruled out. IPNS (*A. nidulans*) was also modified by the deletion of eight residues from its C-terminus and substitution of four other residues at the C-terminus (Fig. 1) to give a protein with a similar truncated C-terminus to that reported for *S. cattleya*. However, the resultant hybrid IPNS (Mcat) was completely inactive with respect to isopenicillin N formation.

CD spectroscopic comparisons of the wild-type, hybrid IPNS (Mcat) and other modified IPNSs showed no differences in secondary structure. Precise reasons for the lack of activity of the hybrid IPNS (Mcat) are presently unclear. The possibility should be considered that the stop codon apparently truncating the *S. cattleya* IPNS sequence is either read through in vivo or is artefactual; a single base change to the published *S. cattleya* IPNS sequence restores a 'typical' C-terminus to the *S. cattleya* enzyme.

Although we have determined the apparent K_m and k_{cat} values for the wild-type IPNS and three modified IPNSs, the interpretation (and accuracy) of the kinetic data is com-

Table 3
Apparent kinetic parameters for wild-type IPNS (*A. nidulans*) and mutants

Enzyme type	V_{max} (nmol/min per mg)	K_m (mM)	k_{cat} (min^{-1})	k_{cat}/K_m (mM^{-1} min^{-1})
Wild type	303 (±30)	1.22	12	9.8
Q330A	144 (±20)	0.72	6	8.3
G329	150 (±12)	0.70	6	8.5
I325	32 (±1)	0.82	1	1.3

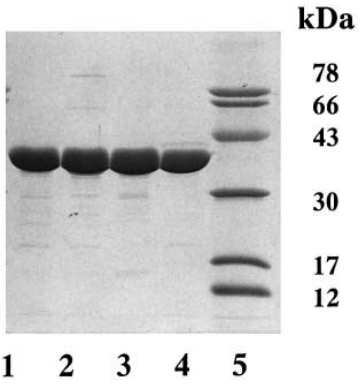

Fig. 2. Coomassie blue stained SDS-PAGE (12.5% gel) analysis of purified mutant and wild-type IPNS (*A. nidulans*). Lanes from left to right show 10 μg of purified enzymes using protocol 2: (1) wt; (2) Q330A; (3) G329; (4) I325; (5) molecular weight markers.

plicated by a number of factors: (1) the lability of the product with respect to hydrolysis; (2) conversion of the substrate thiol to its disulphide (a facile process under the incubation conditions); (3) non-enzymatic consumption of dioxygen (again significant under the incubation conditions); (4) inactivation of IPNS (either via 'incorrect' reaction of catalytic intermediates or by Fenton/Udenfriend type reactions); (5) production of 'shunt products' [1]. Nonetheless the preliminary results based on the analysis of isopenicillin N production are intriguing and merit brief discussion.

The apparent K_m values for ACV for the Q330A, G329 and I325 modified IPNSs are all reduced by similar amounts (approx. 35–45%) relative to the wild-type enzyme. We propose that the side chain of Gln-330 is displaced from the active site iron by the thiol of ACV and the observed reductions in K_m for ACV are consistent with this proposal. Part of the role of the C-terminus may be to maintain ferrous iron at the active site under the relatively low iron concentrations (cf. in vitro incubations) likely to be found in vivo. However, there is also a clear reduction in k_{cat} in the C-terminally modified IPNSs, by approx. 50% in the case of the G330A and G329 mutants and by $>90\%$ in the case of the I325 mutant. These observations imply a role for the C-terminus in steps subsequent to iron and ACV binding. One possibility is that the C-terminus promotes release of the intact penicillin product from the active site; if the penicillin is allowed to remain at the active site the iron may promote Lewis acid catalysed fragmentation of the bicyclic penicillin via complexation to the sulphur of the thiazolidine ring.

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